Variation in EGF/Ras signaling in *C. elegans* and *C. briggsae* vulval development

Honors Research Thesis

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by

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Abstract

The EGF/Ras signal transduction pathway has been shown to be essential in nematode vulval development. When levels of EGF signal are manipulated, precursor cells that give rise to the adult vulva (egg-laying structure) have different division patterns. When EGF/Ras is overactive, the precursor cells experience extra divisions. These extra cellular divisions model tumor growth. Individuals in the human population have varying sensitivities to mutations in this pathway and modern chemotherapy treatments attempt to individualize treatment to account for these differences. To further model this variance, we examine the vulva development system in two species of nematode, *C. elegans* and *C. briggsae*. Like two patients both presenting with tumors, these two species both develop extra cellular divisions from hyperactive EGF/Ras signaling, yet they react differently when treated with drug therapies. I have completed three experiments which show the variation across the two species to U0126, a small molecule inhibitor of mitogen-activated protein kinase kinase (MEK), which acts in the EGF/Ras pathway. The first is a dose response experiment on wild-type animals which shows a complete elimination of vulva development in *C. elegans* but only a partial reduction in development in *C. briggsae*. The second is a drug treatment of nematodes with an extra cell division phenotype which shows elimination of the phenotype in *C. elegans* and a reduced—yet still present—proportion of animals expressing the phenotype in *C. briggsae*. The third experiment exposes the mutant *sur-2*, a downstream component of the EGF/Ras pathway, to the inhibitor and shows complete elimination of precursor cell division in *C. elegans*, and only a marginal decrease in division in *C. briggsae*. The results of these three experiments suggest that the vulval development process in *C. briggsae* is less sensitive to EGF signaling than in *C. elegans* and *C. briggsae* may rely on alternative signaling sources to develop the vulval tissue.

Introduction

The EGF/Ras signal transduction pathway plays important roles in cancer development, and individuals in the population have varying sensitivities to mutations in this pathway (Johnson, 2014). By understanding the underlying molecular basis for cancer development, physicians can alter chemotherapy treatments to fit the unique genetic needs of the patient by altering dosage and targets. This process is still not well understood at such an early point in the field, and thus we need to use model systems for preliminary findings. Studying the vulva development system in two species of nematodes allows us to compare the genetic networks between two species and evaluate the evolution of these networks. Using a model system allows us to manipulate these pathways and develop synthetic mutants. The nematode vulva system is a suitable candidate to answer this research question as there is evidence of a common biological outcome (vulva development) with varying underlying genetic networks between the two species (Felix, 2007).

In signal transduction networks, cells communicate to one another to promote development and spatial patterning. When one cell releases a signaling molecule, often termed a ligand, a neighboring cell receives the signal with a receptor on its surface. This interaction triggers a series of biological events that results in a certain set of genes being turned on within the cell, resulting in a specific physiological response. The nematode egg-laying structure (vulva) develops using several of these networks, including EGF, Wnt, and DSL/Notch. (Yoo, 2004). This thesis focuses on EGF.

The *C. elegans* vulva is comprised of six vulva precursor cells (VPC) located on the ventral surface of the worm. A seventh cell, termed the anchor cell, is dorsal to the vulva precursor cells which divide during development into 22 mature vulval cells. The anchor cell

releases the Epidermal Growth Factor (EGF) ligand where it is subsequently received by the VPCs and influences their cell division (Sommer, 2012). Due to the increased distance between the anchor cell and each individual VPC, the cells receive varying amounts of the EGF ligand and develop into different patterns. The cell located closest to the anchor cell, P6.p, develops into the primary (1^{o}) cell fate, while the two adjacent cells, P5.p and P7.p, develop into the secondary (2^{o}) cell fate. Located even further from the anchor cell are the un-induced cells, P3.p, P4.p and P8.p, which adopt the tertiary (3^{o}) cell fate, divide one time and fuse to the hypodermis. **Figure 1** shows the patterning of a wild-type vulva (Green, 2007).

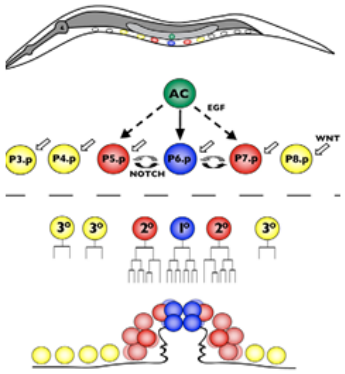


Figure 1: Vulva development system depicting induction of Vulva Precursor Cells (VPCs) P3.p-P8.p by EGF signal released from Anchor Cell (AC) to form 22 adult vulval cells; Lateral inhibition between VPCs depicted with Notch signal; Additional inductive signal depicted with Wnt (Green, 2007)

Two mutant vulval phenotypes discussed in this work are the multivulval (Muv) and vulvaless (Vul). In Muv mutants more vulval precursor cells divide than expected which results in multiple pseudo-vulvae on the ventral surface of the animal. These additional vulvae appear as tumor-like protrusions. The Muv phenotype can be attained when the EGF pathway is hyperactive, causing additional VPCs to divide. In Vul mutants and other mutants with patterning defects, fewer vulval precursor cells divide than expected or the same number of cells divide but take on a lower cell fate (i.e., P6.p attains a 2^{o} cell fate rather than a 1^{o} cell fate). This phenotype can be attained when the EGF pathway is compromised, thus producing less of the EGF ligand to be received by VPCs. This causes them to either remain undivided or to take on a lower cell fate. The wild type, Muv, and Vul mutants are illustrated in **Figure 2**. Examining

these mutants in relation to wild type animals informs our understanding of which signaling pathways are involved in vulval development and to what extent they regulate cell fate decisions.

The egg-laying structure of the nematode worm species *Caenorhabditis elegans* and *Caenorhabditis briggsae* is an appropriate model for studying tissue development. The preliminary data suggest that there is one of two biological phenomena occurring in *C. briggsae* and *C. elegans* vulval development: the *C. briggsae* developmental network is more robust to EGF pathway disruptions, or there is an alternative signaling source that is EGF/Ras-independent involved in *C. briggsae* vulval development. Both questions are pursued in this body of work to uncover pathway flexibilities that might also parallel the variation of individuals in a human population.

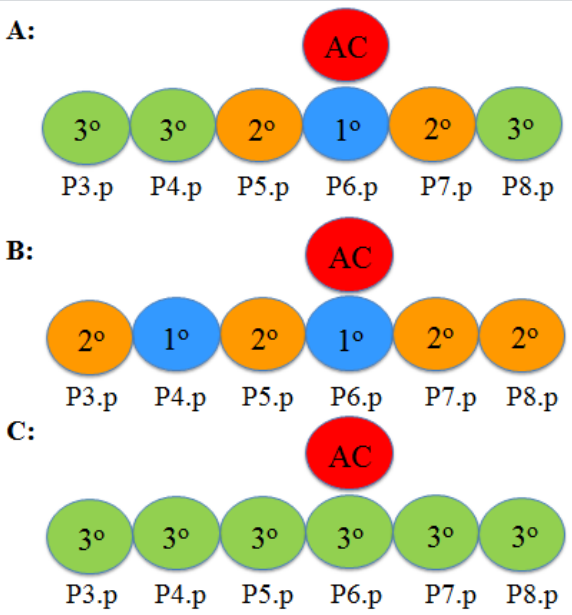


Figure 2: Schematic drawings of VPC cell fates **A:** wild-type animal depicting normal VPC division and cell fates; **B:** Multivulva (Muv) mutant phenotype depicted by over-induction of VPCs which result in multiple ventral protrusions; **C:** Vulvaless (Vul) mutant phenotype depicting under-induction of VPCs which results in no vulva formation

Materials and Methods

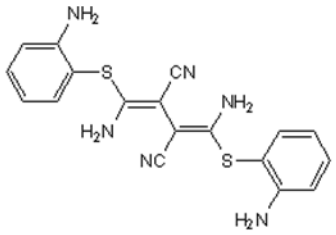
Strains

The Multivulval mutants and the *sur-2* mutant used in these experiments were generated from a genetic screen using Ethyl Methane Sulfonate (EMS). The strains *Cbr-lin-1(gu198)*, *Cbr-lin-31(gu138)*, *Cbr-lin31(gu162)*, *Cbr-pry-1(gu137)*, *Cbr-lin(gu102)*, *Cbr-lin(gu163)*, *Cbr-lin(gu167)*, *Cbr-sur-2(gu185)*, and *Cbr-lin(gu168)* were generated from a genetic screen in the

lab of Dr. Helen Chamberlin. *Cbr-lin-31(sy5342)*, *Cbr-lin-31(sy5344)*, *Cbr-pry-1(sy5353)*, *Cbr-pry-1(sy5411)*, *Cbr-pry-1(sy5270)*, and *Cbr-lin(sy5216)* were generated from the genetic screen in the lab of Dr. Gupta in collaboration with other work between the Chamberlin and Gupta labs. *Cbr-lin-1(sa993)*, *Cbr(bh9)*, *Cel-let-60(n1046)*, and *Cel-sur-2(ku9)* were alleles requested from other labs also researching nematode vulval development. The *C. elegans* and *C. briggsae* wild type strains used were N2 and AF16 respectively.

U0126 assay

C. briggsae and *C. elegans* animals were treated with the MEK inhibitor U0126 (technical data shown in **Figure 3**) using a plate assay, following a general protocol (Reiner et al, 2008). A stock of a 10 mM U0126 solution in DMSO (Dimethyl Sulfoxide) was diluted with 1X M9, with 150 ul of the solution spread on NGM (Nematode Growth Medium) plates (5 ml NGM in 35 mm petri plates). The solution was allowed to absorb into the agar overnight at room temperature, with final concentration of drug calculated for the whole plate volume. Three drops of OP50 *E. coli* were added to the center of each plate on day two, and allowed to dry overnight at room temperature. On day three, approximately 20 L1 worms were added to each plate. All plates were then moved to 20°C. When specific vulval precursor cell fates were determined using cell lineage markers under high magnification, L4 animals were scored two days after originally being plated. When the Muv phenotype was being scored, adult worms were scored for presence of extra ventral protrusions after three days.



U0126

MW:380.48

Formula:$C_{18}H_{16}N_6S_2$

Solubility: DMSO

Figure 3: Technical Data for U0126, MEK Inhibitor (http://www.reagentsdirect.com/index.php/u0126.html)

Microscopy

Animals were scored using both a dissection microscope and a compound microscope with Nomarski to view phenotypes on both the whole-animal and cellular level. When data were collected from animals to determine the proportion of animals who exhibited the Muv phenotype, the dissection microscope was used. Each animal was placed into three categories based on the number of sites on the ventral surface of the worm that had protruding vulval cells, those being zero, one, and greater than one protrusion. When the data were represented in the figures, the one and greater than one protrusion categories were combined and scored as Muv. The zero protrusion category was scored as non-Muv. This was viewed at plate-level in adult animals.

When data were collected from animals to determine the number of vulval precursor cells (VPCs) that divided, the Nomarski compound microscope was used. Mid-L4 animals were mounted on agar pads with 10 mM Sodium Azide anesthetic and viewed at high magnification. The number of divided VPCs was determined by analyzing the tissue morphology under DIC and the *egl-17::GFP* secondary cell lineage marker. Epifluorescence was visualized using GFP filters on the Nomarski compound microscope.

Results

C. briggsae multivulval mutants exhibit varying sensitivities to EGF signal disruption and are less sensitive to pathway interference than *C. elegans*

The multivulval mutant results from a hyperactivated EGF pathway. Both species provided a reliable pool of Muv mutants upon treatment with EMS, which were treated with the U0126 MEK inhibitor. MEK acts in the EGF pathway within vulval precursor cells and is shown

in **Figure 4**. This drug has proven to work in worm species previously (Reiner et al, 2008; Dichtel-Danjoy, 2004). Fourteen lines were generated from the EMS mutagenesis screen. One line from each complementation group was tested using the U0126 inhibitor. *Cbr-lin-1* was tested using the inhibitor as a negative control because of its predicted position in the pathway.

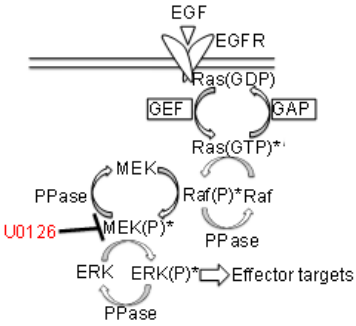


Figure 4: EGF signaling pathway within VPC upon receiving extracellular EGF signal; U0126 depicted in red and acts as a kinase inhibitor drug which perturbates pathway at MEK (MAPKK) element. ETS, the protein produced by *lin-1*, is predicted to act downstream of MEK in this pathway

Figure 5 shows the proportion of animals who confer the Muv phenotype when exposed to 0 uM, 10 uM, and 30 uM U0126 during larval stage development. While *Cbr-lin-1(gu198)* shows no difference between the experimental and control group, all of the other Muv mutants show a decrease in Muv proportion as the dose of the U0126 inhibitor increases. This result suggests that the Muv phenotype in these *C. briggsae* mutants is Mek-dependent.

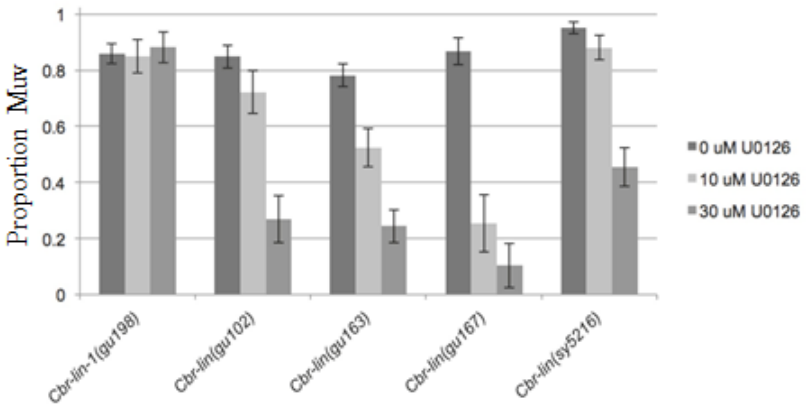


Figure 5: The MEK inhibitor U0126 blocks the Muv phenotype associated with all four *C. briggsae* Muv mutants that define new genes. The Muv phenotype associated with *Cbr-lin-1(gu198)* mutants is not reduced in the presence of U0126, a result consistent with the expectation that *lin-1/Ets* acts downstream of MEK in the EGF pathway. Error bars designate the 95% confidence interval

To compare the sensitivity of MEK inhibition in *C. briggsae* to the sensitivity in *C. elegans*, the U0126 inhibitor was tested on the *C. elegans* Muv mutant *Cel-let-60(n1046)*. *let-60* encodes the Ras protein and acts in the EGF pathway

downstream of the *let-23* EGF Receptor (EGFR) and upstream of MEK. This mutant makes Ras constitutively active. Due to its predicted position in the pathway, *let-60* is expected to be affected by MEK inhibition. **Figure 6** shows the results of testing the inhibitor on the *C. elegans* Muv mutant and *Cbr-lin(gu167)*, which is representative of the *C. briggsae* Muv mutant pool. The *Cbr-lin(gu167)* line was chosen because it showed the most sensitive reaction to the inhibitor. In the assay, the *C. elegans* mutant showed an elimination of the Muv phenotype at the 10 uM concentration while the *C. briggsae* mutant retained the phenotype. This result suggests that EGF signaling is important for vulval development in both *C. elegans* and *C. briggsae*, but may regulate the system less strictly in *C. briggsae* than in *C.* elegans.

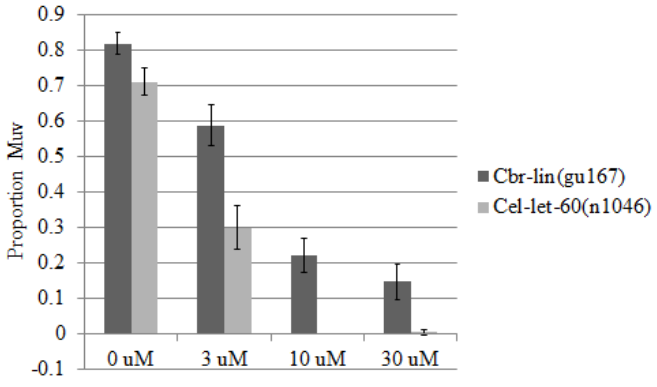


Figure 6: *Cbr-lin(gu167)* serves as representative of *C. briggsae* Muv mutants for comparison to *Cel-let-60(n1046)* Muv mutant. *C. elegans* mutant is more sensitive to MEK inhibition than *C. briggsae* mutants

<u>*C. briggsae* show a lower sensitivity to EGF perturbation than *C. elegans* in an unstressed system</u>

To test the effect of U0126 on *C.* elegans and *C.* briggsae in the absence of other pathway perturbations, the MEK inhibitor was also tested on genetically wild type animals. Since these animals do not exhibit the Muv phenotype, they were assayed for the number of vulval precursor cells which divide upon treatment with the inhibitor drug by examining the cell morphology under high magnification. In a wild type animal three VPCs divide (P5.p, P6.p, and P7.p). Any level of division greater than the 3^{o} cell fate is called induction. In the example of a wild type

animal, three cells are said to be induced. The data from the wild-type assay are shown in **Figure 7**. The *C. elegans* animals displayed eliminated induction at 100 uM U0126. *C. briggsae* animals displayed a reduction, but VPC induction is never eliminated. Doses higher than 100 uM were tested, but no animals survived as adults (data not shown).

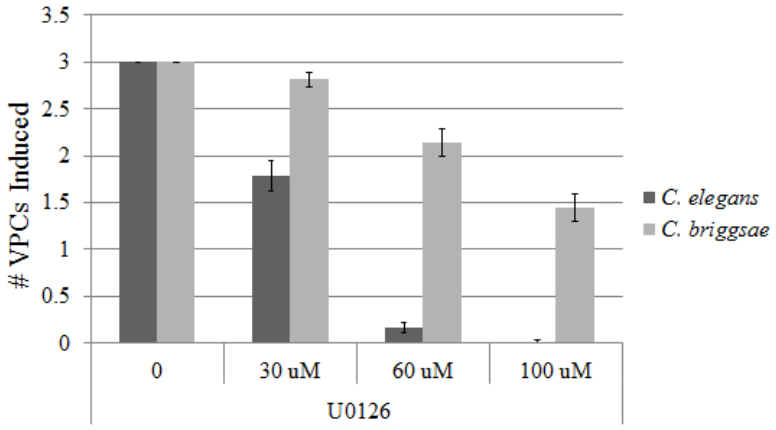


Figure 7: U0126 dose response curve for *C. elegans* and *C. briggsae* wild type nematodes; Induction of VPCs is completely eliminated in *C. elegans*, which mimics the result in the distantly-related species *Oscheius tipulae* (Dichtel-Danjoy; Félix, 2004); Upon same U0126 treatment levels, *C. briggsae* appear to be less sensitive to this type of pathway perturbation; error bars display standard error of the mean

Phenotypic data was also collected from the U0126 wild type assay and animals were scored for presence of the *egl-17::GFP* secondary cell lineage marker. In an untreated wild type animal this marker expresses in the P5.p and P7.p which lie adjacent to the primary cell. Representative images from the assay are shown in **Figure 8**. Untreated wild type animals consistently expressed vulC and vulD cells (secondary cell lineage) in the P5.p and P7.p. Wild type *C. briggsae* animals treated with 30 uM U0126, which had an average of less than two VPCs dividing, expressed the GFP-positive vector in vulC cells in the P6.p. The P6.p traditionally confers the primary cell fate and would not express this reporter.

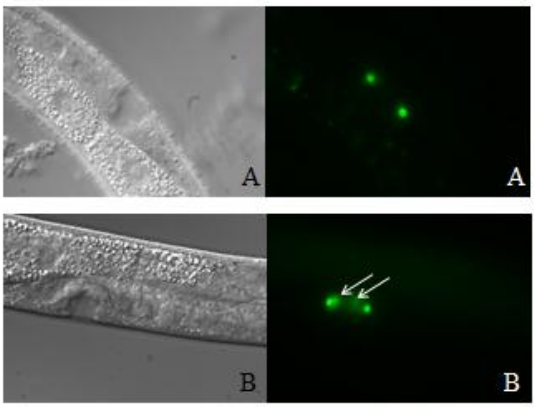


Figure 8: DIC (left) and GFP (right) representative phenotypic data showing loss of primary cell fate in wt P6.p upon treatment with U0126. **A:** *C.br* wt *egl-17::GFP* untreated expressing vulC (secondary cell fate) cells in P5.p and P7.p **B:** *C.br* wt *egl-17::GFP* treated with 30 uM U0126 expressing vulC (secondary cell fate) cells in P6.p, indicated by the two arrows

Both *C. briggsae* and *C. elegans sur-2* mutants show a loss of the primary cell fate with EGF perturbation and *C. briggsae* show a lower sensitivity to the inhibition

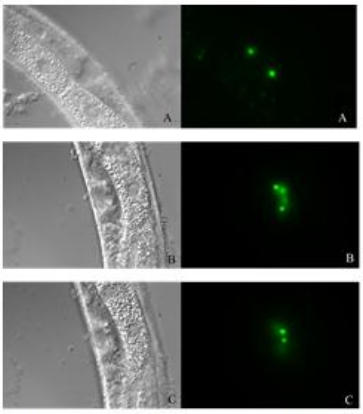


Figure 9: DIC (left) and GFP (right) representative phenotypic data showing loss of primary cell fate in *gu185* P6.p. **A:** *C.br* wt *egl-17::GFP* untreated expressing vulC (secondary cell fate) cells in P5.p and P7.p **B:** *gu185 egl-17::GFP* expressing vulC (secondary cell fate) cells in P6.p; **C:** *gu185 egl-17::GFP* expressing vulE (secondary cell fate) cells in P6.p

The first prediction that the *C. briggsae* developmental network is more robust to EGF pathway disruptions was tested by examining the synergistic effect of *sur-2* mutants treated with U0126. The gene *Cel-sur-2* acts in the EGF/Ras pathway to regulate transcription of vulva development genes downstream of *let-60* Ras and encodes a member of the mediator complex protein MED-23. The mediator complex assembles with RNA polymerase II to regulate transcription of genes important in vulval development (Singh, 1995). When mutated in *C. elegans*, it suppresses the effect of activated Ras protein, thus reducing EGF/Ras signaling by binding a mutant SUR-2 protein with wild-type components of MED-23. This results in a non-functional mediator complex. Since this gene plays an important role in *C. elegans* vulva development (Singh, 1995), it is a suitable candidate for experimentation in *C. briggsae*. In these experiments, the *Cbr-sur-2* mutant (*gu185*) showed a decrease in proportion of animals taking on a 1^{o} cell fate in the P6.p VPC. Phenotypic data are shown in **Figure 9** which reveals untreated *C.br-sur-2* mutants express the *egl-17::GFP* secondary cell lineage marker in the P5.p, P6.p, and P7.p, thus losing primary cell identity in the P6.p.

Since U0126 also disrupts the EGF/Ras signaling pathway at a different target than *sur-2*, coupling its effects with the mutant is predicted to enhance the phenotype, thus confirming the primary role of EGF/Ras in vulva development. Both *Cel-sur-2(ku9)* and *Cbr-sur-2(gu185)* were treated with U0126 and scored for number of VPCs dividing when the cell morphology was viewed under high magnification. As shown in **Figure 10**, induction was eliminated in *C. elegans* and maintained in *C. briggsae* upon treatment with U0126, yet the coupled effect of the MEK inhibitor and the mutant line did not display a significant synergistic effect.

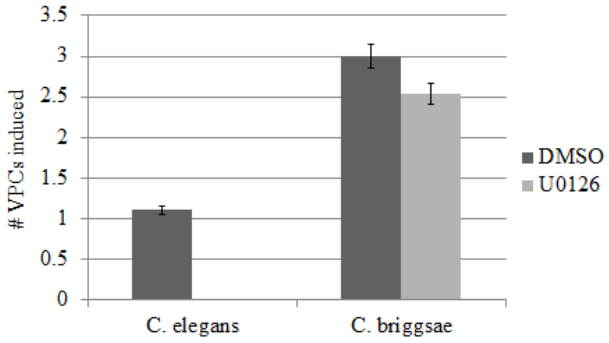


Figure 10: *Cel-sur-2* [*ku9*] is a suppressor of *Cel-let-60* (Ras) and *Cbr-sur-2[gu185]* is a negative regulator of *Cbr-let-60* (Ras); *C. briggsae* show partial reduction in induction and *C. elegans* show complete elimination of induction upon treatment with U0126

<u>Alternative signaling pathways were tested to identify the source of differences in EGF suppression seen between *C. elegans* and *C. briggsae*</u>

To test the second prediction that there is an alternative signaling source that is EGF/Ras-independent involved in *C. briggsae* vulval development, additional signaling sources were tested. Among the candidates were Wnt and Notch. Wnt has been previously understood to sensitize VPCs to EGF signaling and Notch is known to function in lateral inhibition of EGF signaling. One of these signaling sources may serve as the explanation of the differences in vulval induction between *C. elegans* and *C. briggsae*. A vector was created with a truncated form of *Cbr-cam-1* which lacks the transmembrane domain driven by a *myo-3* promoter. *cam-1* encodes a receptor tyrosine kinase which co-localizes with *lin-17* to form the frizzled receptor in

the Wnt signaling pathway on the surface of VPCs. Because the ligand-binding domain is still expressed, this reporter is predicted to sequester Wnt ligands by expressing in the muscle cells surrounding the VPCs and reduces Wnt signaling in the developing vulval tissue (Green, 2007). Lines produced from this reporter showed no observable phenotype in the vulva or gonad and also showed no observable synergistic effect when coupled with the U0126 inhibitor. While this negative result is not conclusive, the VPCs were still primed and able to divide. A second vector was created which promotes the transcription of a truncated form of *sel-8/MAM* upon activation of a heatshock promoter. The SEL-8 protein binds to and stabilizes the DNA-binding complex of the intracellular domains of Notch DNA-binding proteins in the nucleus (Oyama, 2007). This gene serves as a way to reduce Notch signaling within vulval precursor cells by eliminating the domain responsible for recruiting kinase and co-activator domains. The truncated form of the protein creates protein complexes which assemble with other wild-type domains but do not produce a functional protein complex. The table in **Figure 11** displays a low frequency phenomenon where two adjacent VPCs confer a primary cell fate, rather than a primary cell flanked by two secondary cells. A representative image is shown in **Figure 12.** This

	wild-type	mutant	% mutant
RFP-	24	0	0.0%
RFP+	22	11	33.3%

Figure 11: Notch interference driven by heat shock promoter showing adjacent primary cell fate conferring a bivulval phenotype (2°1°1°2°, P4.p-P7.p). Data represent two different lines of *Cbr-sel-8* heatshocked for 60 minutes at 35° C using an RFP promoter, where RFP- serves as the control group

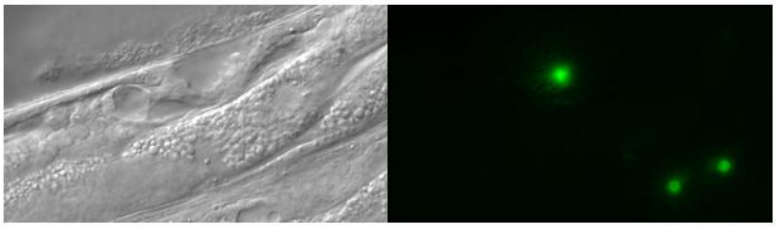


Figure 12: DIC (left) and GFP (right) representative phenotypic data showing abnormal bivulval phenotype (2°1°1°2°). *C.br-sel-8-egl-17::GFP* expressing vulC (secondary cell fate) cells in P5.p only

supports the role in promoting the 2^{o} cell fate which Notch is understood to play based on previous work.

Discussion

Vulval development was measured in three settings where the EGF pathway was hyperactived, unstressed, or compromised. Both wild type and U0126-treated animals were scored in the assays. Across these assays, there were four main conclusions:

1) Vulval development can be eliminated in *C. elegans* upon treatment with an EGF pathway inhibitor. When the *Cel-let-60* mutant was treated with the inhibitor the Muv phenotype was completely eliminated upon increasing the dose of U0126. In the unstressed wild type assay this result was replicated with vulval induction being completely eliminated with the 100 uM U0126 treatment. In the EGF compromised assay *C.el-sur-2* had a basal level of lower induction than wild type *C. elegans* and treatment with U0126 was still able to fully eliminate induction. Collectively, these three assays confirm the dominating role EGF plays in promoting vulval induction in *C. elegans*. Without EGF present, this species is unable to develop the vulva.

2) Vulval induction is only partially eliminated in *C. briggsae* upon treatment with same EGF pathway inhibitor. In the hyperactivated EGF animals the Muv phenotype was maintained across all doses of U0126 tested. While the alleles showed varying sensitivities to MEK inhibition, none of the animals had the phenotype completely eliminated. In the unstressed assay the *C. briggsae* animals showed a consistent decrease in VPC induction but cell division was never eliminated. In the EGF-compromised *sur-2* mutants induction was still not able to be eliminated even with treatment with U0126. These three assays imply that EGF signaling is important for *C. briggsae* vulva development but also that there must be an additional signaling

source that can compensate for EGF when it is compromised. This signaling source may either be novel to *C. briggsae*, or more likely it is a signaling source that is shared among the two species but plays a more functionally-important role in *C. briggsae* than in *C. elegans.*

3) Loss of primary cell fate after U0126 treatment suggests EGF is responsible for vulval precursor cells adopting the primary fate. Regardless of how EGF was compromised across the three assays (MEK inhibition via U0126 or *sur-2* mutants), the *C. briggsae* P6.p cell conferred a secondary cell identity in treated or compromised animals and maintained a primary cell identity in untreated wild type animals. With the only changing variable being the level of EGF activity, the data suggest that EGF is necessary and sufficient to promote a primary cell identity in a vulval precursor cell in *C. briggsae* vulval development.

4) One interpretation of the consistent results in EGF hyperactived, unstressed, and compromised systems suggest *C. briggsae* vulval development may require alternative signaling sources other than EGF which may be Notch. When Wnt signaling was compromised using the *cam-1* reporter there was no observable phenotype either alone or in combination with the U0126 inhibitor. These data do not support that Wnt signaling accounts for the difference between the two species, yet they do not exclude it as a candidate for the difference either. When Notch signaling was compromised using the heat shock *sel-8* vector the *C. briggsae* animals showed adjacent primary cells in VPCs. It is known that EGF and Notch signaling act antagonistically to one another, and the result of adjacent primary VPCs implies that Notch is responsible for conferring the secondary cell identity. Further experiments can be done to see if vulval induction is completely eliminated in *C. briggsae* when both EGF and Notch are compromised.

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